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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 9/12, C12Q 1/48, G01N 33/573, 33/50, A61K 38/45 — A1

(11) International Publication Number: **WO 00/08135**

(43) International Publication Date: 17 February 2000 (17.02.00)

(21) International Application Number: PCT/US99/17724

(22) International Filing Date: 5 August 1999 (05.08.99)

(30) Priority Data:
60/095,976 9 August 1998 (09.08.98) US

(71) Applicant *(for all designated States except US)*: GERON CORPORATION [US/US]; 230 Constitution Drive, Menlo Park, CA 94025 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: WHITE, Michael, A. [US/US]; 4223 Willow Grove Road, Dallas, TX 75220 (US).

(74) Agent: LYON & LYON LLP; Chambers, Daniel, M., Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS AND COMPOSITIONS FOR INHIBITING OR STIMULATING TELOMERASE ASSEMBLY

(57) Abstract

Methods and compositions for assembling active telomerase *in vitro* and in cells, be they in culture or *in vivo*, are provided, as are methods and compositions for inhibiting or enhancing telomerase activity through modulation of telomerase assembly. In certain preferred embodiments, methods are provided for the *in vitro* assembly of a telomerase protein component and a telomerase RNA component, wherein the methods involve the addition of one or more chaperonin molecules, particularly substantially purified or recombinant telomerase chaparonins, which include the proteins hsp40, hsp70, hsp90, p23 and HOP. In such methods, one or more telomerase chaperonins are combined in a reaction mixture that also comprises the catalytic protein and RNA components of telomerase. Screening methods for identifying telomerase assembly and activity inhibitors are also provided, along with methods for stimulating or inhibiting telomerase activity and assembly.

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METHODS AND COMPOSITIONS FOR INHIBITING OR STIMULATING TELOMERASE ASSEMBLY

Field of the Invention

The present invention concerns assembly of the multi-component enzyme telomerase. In particular, this invention relates to methods of identifying or screening for telomerase activity modulators, and to methods and compositions for modulating telomerase activity in a composition or cell (be it in culture or *in vivo*) comprising a telomerase catalytic protein subunit and a telomerase RNA component.

Background of the Invention

The following is a general description of literature relevant to the present invention, although none of the following is admitted to be prior art to the invention by virtue of its inclusion herein. Generally, this material relates to telomerase, a ribonucleoprotein (RNP) complex which functions in cells to maintain the integrity of chromosome ends of eukaryotic cells.

Chromosomes are replicated by DNA polymerases as a necessary prerequisite for almost all eukaryotic cell division. The DNA polymerases responsible for chromosomal replication synthesize DNA in a 5'to 3' direction and require a primer to initiate synthesis. Because of this, the "lagging strand" does not replicate to the very ends of linear chromosomes. The chromosome is thus shortened with every cell division. The ends of chromosomes are called telomeres, and in vertebrates are typically composed of long sequences which contain hundreds of thousands of repeats of short, specific nucleotide sequences. For example, in humans the telomeric repeat consists of the hexanucleotide 5'-TTAGGG-3'. The telomeric repeat sequences for many other organisms are also known. *See* PCT WO 93/23572, for example.

Telomerase activity in a cell is associated with the cell's proliferative capacity. *See* U.S. patent nos 5,646,245 and 5,645,986. All normal diploid, somatic vertebrate cells lack telomerase activity and have a limited capacity to proliferate, a phenomenon known as the Hayflick limit or replicative senescence (*i.e.*, loss of the ability of a cell to replicate in the presence of normally appropriate replicative signals). For example, in human fibroblasts, this limit occurs after 50-100 population doublings, after

which the cells remain in a viable but non-dividing senescent state for many months. Typically, about 50-200 or more nucleotides are lost from the ends of telomeres during each cell division. *See* Harley, *et al.*, 1990, *Nature* 345:458-460. Such shortening occurs both *in vivo* and *in vitro*. This contrasts with the behavior of germline and stem cells (and the cells of many lower eukaryotes, *e.g.*, yeast), which have long telomeres and active telomerase. It has also been observed that neoplastic cells (*e.g.*, cancer cells), which have escaped from the controls limiting their proliferative capacity, are immortal. These cells have regained telomerase activity and thus can maintain telomere length, although the telomeres of such cells are typically much shorter than those of corresponding embryonic, germline, or stem cells. For example, telomerase activity has been detected in a diverse set of tumor tissues and in immortal cell lines, but is not detected in assays of most normal somatic cells. *See*, Shay and Bacchetti, 1997, *Eur. J. Cancer*, 33:777-791, and Morin, 1989, *Cell* 59:521. Moreover, increasing levels of active telomerase in telomerase-negative cells results in dramatically increased proliferative capacity. Bodnar, *et al.*, 1998, *Science* 279:349-352.

The enzyme telomerase acts as a specialized reverse transcriptase to synthesize telomeric repeat sequences at the 3' end of the telomere, thus extending the DNA to prevent loss of telomeric sequence, thereby preventing telomere shortening. The telomerase RNP includes a catalytic protein subunit referred to as "TERT" (for telomerase reverse transcriptase) and a telomerase RNA component (referred to herein as "TR"). The telomerase RNA component acts as a reverse transcriptase template for the catalytic protein. TERTs and TRs from multiple species have been cloned and characterized. Human and non-human TERTs are described in, *e.g.*, PCT Publications WO 98/14592 and WO 98/14593; Nakamura, *et al.*, 1997, *Science* 277:955-959; Meyerson, *et al.*, 1997, *Cell* 90:785-795; Lingner, *et al.*, *Science* 276:561-567; and Greenberg, *et al.*, 1998, *Oncogene* 16:1723-1730. The RNA component of telomerases have also been characterized in several species, as described in, *e.g.*, U.S. Patent No. 5,583,016, Feng, *et al.*, 1995, *Science* 269:1236-1241; Blasco, *et al.*, 1995, *Science* 269:1267-1270; and Greider, *et al.*, 1989, *Nature* 337:331-337. In addition to TERT and TR, additional telomerase-associated and telomere-associated proteins have been reported, some of which may have a structural or regulatory function (*see, e.g.*, Harrington, *et al.*, 1997, *Science* 275:973-977; Collins, *et al.*, 1995, *Cell*, 81:677; Nakayama, *et al.*, 1997, *Cell* 88:1-20; U.S. Pat. No. 5,770,422; and PCT publication WO 98/14593 at page 70).

Telomerase activity can be generated in cell-free systems. *In vitro* transcription and translation of human TERT ("hTERT"), when co-synthesized or mixed with the human telomerase RNA ("hTR"), results in activity characteristic of native human telomerase. *See, e.g.*, Weinrich, *et al.*, 1997, *Nature Genetics* 17:498-502.

Because telomerase plays a role in controlling cell proliferation, telomerase is an ideal target for diagnosing and treating diseases relating to cellular hyperproliferation, of which cancer is an example. Indeed, telomerasec activity has been detected in over 98% of immortal cell lines and in more than 85% of malignant tumor biopsies. Wright, *et al.* (1996), *EMBO J.*, vol. 16. no. 17:1734-1741. In addition, activation of telomerase is an exciting approach to diseases and conditions associated with senescence, including aging and age-related processes. Despite the attractiveness of telomerase as a target for limiting or expanding cellular proliferative capacity, the need exists to identify compounds that inhibit or stimulate telomerase activity. This invention addresses this need by providing methods and compositions for identifying molecules that assist and/or participate in the formation of biologically active telomerase holoenzyme complexes, as well as compounds that interact with such molecules.

DEFINITIONS

The following terms shall have the following definitions when used herein, unless otherwise stated.

"Telomerase RNA component" refers to the RNA molecule which is incorporated into the telomerase RNP and required for biological activity. Preferably, the RNA component used is from the same species as the telomerase catalytic protein component, *e.g.*, the human telomerase component is used in conjunction with the human telomerase catalytic protein component. It is also preferred to use a telomerase component comprising a nucleotide sequence which has the same nucleotide as the naturally occurring version of that RNA. However, in certain embodiments, the telomerase RNA component employed may contain at least one nucleotide substitution, insertion, or deletion as compared to the corresponding naturally occurring RNA, be it inside and/or outside of the telomeric repeat template region of the RNA. Indeed, any RNA component which serves as a template for synthesis of a telomeric repeat once assembled into an active telomerase RNP complex can be used in accordance with the present invention. As those in the art will appreciate, such RNAs can readily be identified by incorporating the

same into a telomerase RNA and testing, preferably *in vitro*, whether telomerase RNP complexes containing the same exhibit telomerase activity.

As used herein, the term "telomerase activity" refers specifically to telomerase activity, preferably the processive catalytic activity of telomerase as described in WO 98/14593. Other activities of telomerase which may be screened include non-processive catalytic activity, reverse transcriptase activity, nucleolytic activity, primerr or substrate binding activity, RNA (*i.e.*, TR) binding activity, and protein binding activity. Telomerase processive catalytic activity is characterized by the ability of the telomerase RNP to extend a DNA primer that functions as a telomerase substrate by adding multiple (*i.e.*, at least two) repeats of a sequence (*e.g.*, 5'-TTAGGG-3') encoded by the template nucleic acid (*e.g.*, hTR). This activity can be assayed, e.g., as described in Morin, 1989, *Cell* 59:521 ("conventional assay") or using the TRAP assay disclosed in U.S. Patent No. 5,629,154.

As used herein, the term "telomerase assembly" refers to the formation of a ribonucleoprotein complex including the telomerase catalytic subunit protein (TERT) and a template RNA (*e.g.*, TR) and having telomerase activity. Thus, a compound or treatment that stimulates telomerase assembly will increase telomerase activity. Conversely, a compound or treatment that inhibits telomerase assembly will decrease telomerase activity.

As used herein, "*in vitro* assembly" refers to the generation of telomerase activity *in vitro* by combining (*e.g.*, by coexpression, mixing, or a combination thereof) a TERT polypeptide, preferably a recombinant form thereof, with a synthetic or recombinantly produced telomerase RNA component (*e.g.*, hTR) or a functionally equivalent template RNA. *In vitro* assembly is described generally in PCT Publication WO 98/14593, and in Weinrich et al., *supra. Also see,* Beattie *et al.*, 1998, *Curr. Biol.* 8:177-180. According to one method, *in vitro* assembly is accomplished by coexpression of the TERT polypeptide and a telomerase RNA in a cell-free transcription-translation system (*e.g.*, a wheat germ or rabbit reticulocyte lysate). In an alternative embodiment, TERT and the RNA component are separately expressed (and preferably one or both are partially or completely purified) and then mixed to achieve assembly of an active telomerase RNP. One of the discoveries disclosed herein is that assembly of TERT and TR in the presence of "telomerase assembly related proteins," or "telomerase

chaparonins," results in substantially more efficient telomerase assembly and activity than assembly in the absence of such proteins.

As used herein, "telomerase assembly related proteins" or "telomerase chaparonins" are proteins that facilitate the association of TERT and TR in assembly of the telomerase RNP. As those in the art will appreciate, a telomerase chaparonin is a protein that binds to or is associated with unfolded or partially folded telomerase protein component to maintain their unfolded or partially folded state to ensure proper folding or assembly into multi-subunit complexes, *e.g.* telomerase. These "chaparonins" or "assembly related proteins" include, but are not limited to, phosphoprotein 23, heat shock proteins 40, 70, and 90, and HOP (also called p60). The human homolog of each of the foregoing proteins is described in additional detail below. The human form of these proteins is preferred in various embodiments of the present invention, particularly those in which the TERT is hTERT and the TR is hTR. However, it will be recognized that the present invention is not limited to the use of human sequence proteins. Telomerase chaparonins from non-human species (*e.g.*, mice and yeast) are publicly available and/or can be obtained by one of ordinary skill using routine techniques. It should also be recoginized that compounds other than proteins may exhibit telomerase chaparonin-like activity. For example, as part of this invention, it is been determined that molybdate can substitute for one or more of the telomerase chaparonins required assembly of the telomerase RNP. Thus, the present invention also contemplates that non-polypeptide compounds which stabilize the same conformational positions as a polypeptide telomerase chaparonin will be considered a telomerase chaparonin for purposes of this invention. Such compounds can be identified using techniques known in the art, for example, by screening various compounds, preferably in a high throughput format, to determine whether they enhance or inihibit functional telomerase RNP assembly in an *in vitro* assembly reaction.

a) Phosphoprotein p23 ("p23")

As used herein, "p23" refers to an approximately 23 kDa phosphoprotein. The sequence of human p23 protein is provided in GenBank Accession No. 1362904 and is encoded by a gene having the sequence of GenBank Accession No. Z69891. The bacterial expression and purification of human p23 has been described (*see, e.g.*, Johnson *et al.*, 1994, *J. Biol. Chem.* 269:24989). Briefly, the soluble fraction of bacterial lysate

derived from bacterial cells engineered to express the protein was fractionated by DEAE-cellulose column chromatography, followed by phenyl-sepharose (hp1660) FPLC, dialyzed into 10 mM Tris-HCl, 1 mM DTT and 1 mM EDTA, pH 7.5, and stored at -70°C. The preparation was greater than 99% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration can be determined by amino acid analysis.

b) Heat shock protein hsp90 ("hsp90")

As used herein, "hsp90" refers to an abundant heat shock phosphoprotein found in the cytosols of animal and plant cells and having an approximate molecular weight of 90 kDa; the sequence of human hsp90β protein is provided in GenBank Accession No. J04988 and is encoded by a gene having the sequence of GenBank Accession No. X15183 . The bacterial expression and purification of human hsp90 has been described (*see* Sullivan, *et al.*, J.Biol.Chem. 272:8007). Briefly, human HSP90β was overexpressed in SF9 cells. The protein was purified from cytosol extracts to greater than 99% purity by chromatography on columns of DEAE-cellulose, heparin-agarose, and mono Q. Purified hsp90 was stored at -70°C in 10 mM Tris-HCl, 100 mM KCl, 1 mM EDTA and 10% glycerol, pH 7.4.

c) Heat shock protein hsp40 ("hsp40")

As used herein, "hsp40" refers to an approximately 40 kDa phosphoprotein. The sequence of human hsp40 protein is provided in GenBank Accession No. 1816452, and is encoded by a gene having the sequence of GenBank Accession No. 085492. The bacterial expression and purification of Ydj1p, the yeast homolog of human hsp40, was performed by fractionating bacterial cell lysates engineered to overexpress the protein by DEAE-cellulose chromatography followed by hydroxylapatite column chromatography. The preparation was approximately 80% pure as assessed by densitometry of SDS-PAGE. Protein concentration can be determined by amino acid analysis.

d) Heat shock protein hsp70 ("hsp70")

As used herein, "hsp70" refers to an approximately 70 kDa phosphoprotein. The sequence of human hsp70 protein is provided in GenBank Accession No. 292160, and is encoded by a gene having the sequence of GenBank Accession No. L12723 . The bacterial expression of human hsp70 employed the same system as used for expression of p23. Briefly, hsp70 was prepared by the overexpression in SF9 cells. The protein was

then purified by fractionating cell lysates by DEAE-cellulose column chromatography followed by ATP-agarose column chromatography. Protein was precipitated using ammonium sulfate (75% saturation), and the redissolved hsp70 was fractionated by 16/60 Superdex 200 FPLC. Only the monomer peak of hsp70 was retained. The preparation was approximately 97% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration can be determined by amino acid analysis.

e) HSP Organizing Protein (HOP or p60)

As used herein, "HOP" or "p60" refers to an abundant, stress-induced protein found in certain cells. The sequence of human HOP protein is provided in GenBank Accession No. 306890 and is encoded by a gene having the sequence of GenBank Accession No. M34664. The bacterial expression and purification of human HOP has been described by Schumacher et al. 1994, *J. Biol. Chem.* 269:9493-9499. Briefly, human Hop was expressed in bacteria. Bacterial lysates were then fractionated by DEAE-cellulose chromatography followed by hydroxylapatite column chromatography. Additional purification was achieved by fractionating the pool from hydroxylapatite on a Mono Q FPLC column (10/10, Pharmacia) which was eluted with a linear gradient of 0-0.5 M KCL. The fractions containing Hop were pooled, dialyzed into 10 mM Tris-HC1, 1 mM DTT and 1 mM EDTA, pH 7.5 and stored at -70°C. The preparation was approximately 94% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration can be determined by amino acid analysis.

As used herein, "modulate," in the context of modulating telomerase activity or assembly in a cell, cell lysate, *in vitro* composition, or cell (in culture or *in vivo*) refers to the inhibition or stimulation of telomerase activity or assembly. The term "modulator" refers to any synthetic or natural compound or composition that can modulate telomerase assembly or activity. A modulator can be any organic and inorganic compound, including, but not limited to, for example, small molecules, peptides, proteins, sugars, nucleic acids, fatty acids and the like.

As used herein, the term "substantially pure," or "substantially purified," when referring to a composition comprising a specified reagent such as a telomerase assembly associated protein means that the specified reagent is at least about 75%, or at

least about 90%, or at least about 95%, or at least about 99% or more of the composition (not including, *e.g.*, solvent or buffer).

As used herein, "recombinant" refers to a polynucleotide synthesized or otherwise manipulated *in vitro* (*e.g.*, "recombinant polynucleotide"), to methods of using recombinant polynucleotides to produce gene products in cells or other biological systems, or to a polypeptide ("recombinant protein") encoded by a recombinant polynucleotide.

Brief Summary of the Invention

The objects of this invention include the provision of novel and non-obvious methods of screening for compounds that modulate telomerase activity, methods of assembling telomerase, as well as methods and compositions for modulating telomerase activity *in vitro* and *in vivo*.

One aspect of the invention provides for methods of screening for telomerase activity modulators (*i.e.*, compounds which inhibit or stimulate telomerase activity) by assembling telomerase in the presence or absence of a test compound. Telomerase activity is then measured and compared with the telomerase activity detected when telomerase is assembled in the absence of the test compound, and test compounds which increase or decrease telomerase activity are identified as telomerase activity modulators.

In another aspect, methods are provided for identifying chaparonins which facilitate assembly of active telomerase. In certain embodiments, these methods are performed *in vitro*, while in other embodiments, the methods are performed *in vivo*. In certain preferred embodiments, a telomerase catalytic protein component and a telomerase RNA component are added to reaction mixture comprising at least one polypeptide, preferably at least one putative chaparonin, particularly a chaparonin selected from the group consisting of p23, hsp40, hsp70, hsp90, and HOP. Of course, one or more other chaperonins can also be included or substituted in such reactions, including, non-polypeptide-based chaperonins. Telomerase activity of the reaction mixture is then compared to a reaction mixture control. Preferably, at least one, and preferably each, of the catalytic protein component, the telomerase RNA component, and polypeptide(s) are substantially purified, preferably from either a recombinant or natural source. In other embodiments, one or more of the catalytic protein component, the telomerase RNA component, and polypeptide(s), particularly the catalytic protein component and/or

telomerase RNA component, are also transcribed and/or translated, as the case may be, in the reaction mixture. In particularly preferred embodiments of this and other aspects of the invention, the catalytic protein component, the telomerase RNA component, and/or the chaparonin(s) are/is a human telomerase component or chaparonin, and can be derived from either a natural or recombinant source.

A related aspect of the invention concerns the assembly of telomerase in the presence of one or more chaparonins *in vitro* or *in vivo*. In certain preferred *in vitro* embodiments, the catalytic protein component and the RNA component of telomerase are added to a mixture comprising at least one chaparonin. In other preferred embodiments, a chaparonin (or an expression vector encoding the same) is introduced into a cell which expresses the catalytic protein and RNA components of telomerase, thereby, for example, to increase or enhance the telomerase activity, preferably to extend its replicative capacity.

Another related aspect of the invention concerns *in vitro* and *in vivo* methods of screening for telomerase activity modulators which act by modulating chaparonin-mediated assembly of telomerase. Certain preferred *in vitro* embodiments of this aspect relate to methods where the catalytic protein and RNA components of telomerase are assembled in a reaction mixture comprising at least one chaparonin in the presence or absence of a test compound. High throughput screening method formats are well known in the art. Typically, a number of different compounds (from as few as about 10-100 to 10,000 or more) are individually, and frequently simulataneously, screened in separate reactions, are particularly preferred. Telomerase activity is then measured and compared with the telomerase activity in a control reaction mixture lacking the test compound, and test compounds which increase or decrease telomerase activity are identified as telomerase activity modulators. In other preferred embodiments, test compounds are screened against cells known to possess telomerase activity, for example, an immortal cell line. Compounds found to confer mortality on such cells can then be identified. The use of such cell based assays is particualrly preferred to confirm modulating activity that has first been identified in an *in vitro* telomerase assembly screening assay.

In a yet another related aspect, the invention provides methods of enhancing telomerase activity *in vitro* or *in vivo* by providing at least one chaparonin to facilitate the assembly of TERT and TR into biologically active telomerase RNP complexes. In certain preferred *in vitro* embodiments of this aspect, the chaparonin is

selected from the group consisting of p23, hsp40, hsp70, hsp90, and HOP, with the chaparonin being a substantially purified natural or recombinant protein in particularly preferred embodiment. In other embodiments, the *in vitro* assembly of the catalyitc protein component and telomerase RNA component into biologically functional RNP complexes occurs in a cell extract-based translation system, *e.g.*, a rabbit reticulocyte lysate or a wheat germ extract. In such systems, RNA coding for one or more of the catalyitc protein component and chaparonin(s) are added to the translation system. In other embodiments, the translation system employed comprises only defined constituents, as opposed to being crude cell extracts. Yet other embodiments of this aspect may be conducted *in vivo*.

In another aspect, the invention provides a method of screening for telomerase assembly inhibitors by contacting (incubating) one or more telomerase chaparonins (*e.g.*, p23, hsp90, hsp40, hsp70, and HOP) and TERT in the presence or absence of a test compound; comparing the association of the chaparonin(s) and TERT in the presence of the test compound with association in the absence of the test compound, and identifying a compound that decreases the association of the chaparonin(s) and TERT as a telomerase assembly inhibitor.

Yet another aspect of the invention concerns methods of modulating telomerase activity in a cell expressing a telomerase catalyitc protein component and a telomerase RNA component. Certain preferred embodiments of this aspect concern decreasing the amount of a telomerase chaparonin in the cell; inhibiting association of a telomerase chaparonin with a telomerase catalytic protein component; inhibiting association of a telomerase chaparonin with a telomerase RNA component; stimulating association of a telomerase chaparonin with a telomerase catalytic protein component; stimulating association of a telomerase chaparonin with a telomerase RNA component; and increasing the amount of a telomerase chaparonin in the cell. Each of these embodiments involves administration of an amount of a compound effective to modulate telomerase activity of the cell. In some embodiments of this aspect, as well as other aspects of the invention, the cell to be treated is a vertebrate cell, preferably an animal cell, for example an avian, bovine, canine, equine, feline, ovine, or porcine cell. Particularly preferred cells are mammalian cells, including human cells, be they in culture or *in vivo*.

In preferred embodiments of all aspects of the invention, the compound, including the test compound, is a small molecule (*i.e.*, an organic molecule, other than

nucleic acid or polypeptide, that has a molecular weight of less than about 10kD, preferably less than about 5kD, particularly less than 1.5kD), a nucleic acid (*i.e.*, a synthetic or natural single or double-stranded polynucleotide comprising more than about six nucleotides), or a polypeptide (*i.e.*, two or more amino acids joined by a peptide bond, including proteins, enzymes, and fragments thereof). Particularly preferred nucleic acids are expression vectors, antisense nucleic acids (DNA or RNA), oligonucleotides, and a ribozyme.

Yet another aspect of the invention concerns pharmaceutical compositions which contain a therapeutically effective amount (*e.g.*, the amount required to achieve the desired therapeutic effect, which will depend on the patient and condition to be treated, and will preferably be an optimized amount such that the desired modulating effect (inhibition or stimulation, as the case may be) is achieved without significant side-effects to the extent that those can be avoided, but to the extent they can not be or are otherwise tolerable, the minimum amount necessary to achieve the desired modulation) of a compound which modulates telomerase activity through modulation of the association between one or more telomerase chaparonins and the telomerase catalytic protein component and/or a telomerase RNA component of the telomerase holoenzyme. Also included in the compositions is a pharmaceutically or veterinarianally acceptable carrier. When the composition contains a nucleic acid, the formulation may include one or more targeting elements. Other compounds may also be included in the formulations of the invention, such as excipients, buffers, *etc.* Such compositions include those which are in liquid or dry (*e.g.*, freeze dried and lyophilized) form. Such compositions may also be formulated as pills, such as for oral administration. Other routes of administration of various embodiments include by parenteral (*e.g.*, intravenous, intramuscular, and interperitoneal) injection, by inhalation, as well as other routes known in the art. The dosage and treatment regimen for a particular composition will depend on many factors, including the disease or condition to be treated, the age, weight, gender, and physical condition of the patient, *etc.*

Yet another aspect of the invention involves methods of modulating proliferation of cells by administering to cells which contain telomeres an amount of a compound which modulates telomerase activity through modulation of association between a telomerase chaparonin and a telomerase catalytic protein component and/or a telomerase RNA component. According to such methods, the proliferative capacity of

cells can be increased or decreased, depending upon the compound employed, and thus can be used to treat a wide variety of diseases, disorders, and conditions, for example, diseases associated with elevated levels of telomerase activity, *e.g.* cancer, leukemia, and infection with a eukaryotic pathogen, as well as states where telomerase activity is not detectable or is insufficient to prevent cellular senescence, for example, aging, expansion of somatic cells in culture, *etc.*

Brief Description of the Drawings

There are no drawings.

Detailed Description

Introduction

The present invention is based, in part, on the discovery, described *below* in the Examples, that certain telomerase chaparonins, *e.g.*, phosphoprotein p23 (hereinafter "p23") and the heat shock protein hsp90 (hereinafter "hsp90"), associate *in vivo* and *in vitro* with hTERT and can act to stimulate telomerase activity *in vitro*. Without intending to be bound by any particular mechanism, it is likely that these proteins facilitate the association of TERT and telomerase RNA to form a catalytically active telomerase RNP complex (*e.g.*, by maintenance of TERT in a conformation that productively interacts with the telomerase RNA component). A possibly similar interaction has been reported for the association of hsp90/p23 with hepadnavirus reverse transcriptases (Hu et al., 1997, *EMBO* 16:59-68, Hu, et al., 1996, *Proc. Natl. Acad. Sci. USA* 93:1060-1064) and other proteins (Pratt et al., 1997, *Endocrine Reviews* 18:306-360).

The instant invention provides novel methods and compositions for inhibiting or stimulating telomerase activity in a cell (in culture or *in vivo*) or *in vitro* composition to inihibit or stimulate, as the case may be, telomerase RNP assembly. The present invention further provides screening methods for identifying novel compounds that inhibit or stimulate telomerase assembly and activity in cells or *in vitro*. The invention further comprises methods of *in vitro* assembly of active telomerase. In preferred embodiments, such methods comprise combining TERT, TR, and p23, hsp40, hsp70, hsp90, and HOP proteins, particularly where one or more of the components of the reaction mixture are substantially purified, especially purified recombinant proteins.

Assembly

The ability to assemble active telomerase RNP *in vitro* using recombinantly produced TERT has provides an extremely valuable system useful for, *inter alia*, identifying telomerase activity modulators with therapeutic (*e.g.*, anticancer) activity. In one aspect the present invention provides an improved method for *in vitro* assembly of telomerase comprising combining TERT, a telomerase RNA component, and one or more of substantially purified or recombinant telomerase chaparonins p23, hsp40, hsp70, hsp90, and HOP protein.

The present invention encompasses several different ways of combining (or contacting) telomerase assembly associated proteins, TERT, and template RNA (*e.g.*, hTR) to reconstitute active telomerase. In certain embodiments, for example, each of the components is separately produced and purified, and the purified components combined in a reaction mixture containing other constituents required for activity of the protein and/or nucleic acid components (*e.g.*, typically in the presence of buffer, salt, protease inhibitors, RNAse inhibitors, ATP, Mg^{2+}, and the like; *see* Dittmar, *et al.*, 1996, *J. Biol. Chem.* 271:12833-12839, and Dittmar, *et al.*, 1997, *J. Biol. Chem.* 272, 13047-13054). In alternative embodiments, one or more of the components is added in the form of a polynucleotide (*e.g.*, RNA or DNA) comprising a sequence encoding the protein or RNA, which sequence is operably linked to appropriate regulatory elements such as a promoter, under conditions that permit the transcription and/or translation of the protein or RNA. Thus, in one embodiment, expression vectors encoding one or more of the protein and RNA components is incubated in a cell-free expression system. For example (by way of illustration but not limitation), an expression vector encoding hTR (*e.g.*, U.S. Patent No. 5,583,016) can be combined with substantially purified hTERT, p23, hsp40, hsp70, hsp90 and HOP protein to produce or enhance telomerase assembly and activity.

It will be appreciated that *in vitro* telomerase assembly by coexpression of TERT and TR in a rabbit reticulocyte lysate ("RRL") or other cell-free expression system (*e.g.*, wheat germ) can be readily accomplished using art known methods. Some such systems (*e.g.*, the RRL) comprise a complex, undefined (or largely undefined) mixture of proteins and other molecules. Based on the discoveries discussed herein, presumably such systems include at least those telomerase chaparonins (or their functional equivalents) p23, hsp40, hsp70, hsp90 and HOP, and possibly including polynucleotides encoding one or more of these proteins. Insofar as the rabbit reticulocyte lysate or other such cell-free

lysate systems do not include the requisite telomerase chaparonins (perhaps due to pretreatment, *e.g.*, immunodepletion) required for efficient telomerase assembly, as described in the examples, the present invention also contemplates the addition of one, several, or all of the telomerase assembly proteins, preferably in substantially purified, especially recombinant, form to a composition such as a rabbit reticulocyte lysate to increase or stimulate telomerase assembly and activity. For example, in some embodiments, the invention provides methods of enhancing telomerase activity *in vitro* comprising adding at least one substantially purified or recombinant telomerase chaparonin protein, particularly one or more proteins selected from the group consisting of p23, hsp40, hsp70, hsp90, and HOP, to a composition (which may include components of a, *e.g.*, reticulocyte lysate), comprising TERT and a telomerase RNA template.

The sequence of combination of the TERT, RNA, and telomerase chaparonin(s) may be selected based on the convenience of the practitioner. Thus, in some embodiments, for example, the TERT, RNA, and telomerase chaparonin(s) are combined at the same time. In other alternative embodiments, the TERT protein and telomerase chaparonin(s) are combined, and the RNA component is added subsequently.

In certain embodiments, the TERT, RNA, and chaparonin(s) are human proteins (*i.e.*, they have the sequence of naturally occurring human proteins such as those described herein). In other embodiments, one or more of the chaparonins is a functionally active variant or fragment of the human proteins. As used in this context, a variant of hsp90, hsp70, HOP or p23 is "functionally active" if it is able to convert the glucocorticoid receptor hormone binding domain from a non-steroid binding to a steroid binding confirmation, when combined with the other three components (*see* Dittmar, *et al.*, 1996, *J. Biol. Chem.* 271:12833-12839, and Dittmar, *et al.*, 1997, *J. Biol. Chem.* 272, 13047-13054). Typically a variant has at least about 80%, about 90%, or about 95% sequence identity to the naturally occurring protein. In certain alternative embodiments, the chaparonins are from species other than human, such as yeast, mouse, non-human primate, or plant, while in other embodiments, the chaperonins are non-polypeptide-based molecules that faciltate assembly of multi-subunit protein complexes and/or assumption and/or maintenance of a correctly folded three-dimensional conformation, *i.e.*, a conformation that mimics the natural conformation of the protein, thereby enabling it to perform the appropriate biological function(s).

In some embodiments of the invention, the *in vitro* reconstitution of telomerase includes the addition of additional substantially purified or recombinant proteins such as HIP (hsp70 interacting protein, also called p48; *see* Chen, *et al.*, 1996, *Mol. Endocrinol*, 10:682-693; Smith, *et al.*, 1993, *Mol. Cell. Biol.* 13:869-876), other immunophilins (Pratt, *et al.*, 1997, *Endocrine Reviews* 18:306-360; Freeman, *et al.*, 1996, *Science* 274:1718-1720), and others.

In vitro assembled telomerase made according to the methods described herein is useful, *inter alia*, for the screening of telomerase inhibitory and stimulatory agents. Suitable screening assays are known in the art and are described, for example, in WO 98/14593, WO 93/23572, and U.S. Patent No. 5,629,154. As those in the art will appreciate, such assays can be readily adapted for use in the practice of this invention given the disclosure herein. Thus, in one suitable assay (described for illustration and not limitation), telomerase is reconstituted by combining hTERT, hTR, and substantially purified or recombinant telomerase assembly associated proteins (*e.g.*, in a volume of 50 µl). The activity of the *in vitro* assembled telomerase RNP is assayed in the presence and absence of multiple concentrations of test compounds solubilized in DMSO (*e.g.*, 10 µM - 100 µM). In a representative example of such as assay, test compounds (*e.g.*, any synthetic or natural compound or composition, including small molecules, peptides, proteins, nucleic acids, sugars, nucleic acids, fatty acids) are preincubated in a total volume of 25 µL for 30 minutes at room temperature in the presence of 2.5 µL of a solution constituting the components required for telomerase assembly, 2.5% DMSO, and 1X TRAP Buffer (20 mM Tris-HCl, pH 8.3, 1.5mM $MgCl_2$, 63 mM KCl, 0.05%Tween20, 1.0 mM EGTA, 0.1 mg/ml Bovine serum albumin). Following the preincubation, 25 µL of the TRAP assay reaction mixture is added to each sample. The TRAP assay reaction mixture is composed of 1X TRAP buffer, 50µL dNTP, 2.0 µg/ml primer ACX, 4 µg/ml primer U2, 0.8 attomol/ml TSU2, 2 units/50µl Taq polymerase (Perkin Elmer), and 2 µg/ml [^{32}P]5'end-labeled primer TS (3000Ci/mmol). The reaction tubes are then placed in the PCR thermocycler (MJ Research) and PCR is performed as follows: 60 min at 30°C, 20 cycles of (30 sec at 94°C, 30 sec. at 60°C, 30 sec. at 72°C), 1 min at 72°C, cool down to 10°C. The TRAP assay is described, as noted *supra*, in U.S. Patent No. 5,629,154. The primers and substrate used have the sequences: TS Primer (5'-AATCCGTCGAGCAGAGTT-3'); ACX Primer (5'-GCGCGG[CTTACC]3CTAACC-

3'); U2 primer (5'-ATCGCTTCT CGGCCTTTT-3'); TSU2 (5'-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCG AT-3').

After completion of the PCR step, 4 µl of 10X loading buffer containing bromophenol blue is added to each reaction tube and products (20 µl) are run on a 12.5% non-denaturing PAGE in 0.5X TBE at 400 V. The completed gel is subsequently dried and the TRAP products are visualized by a phosphorimager or by autoradiography. The telomerase activity in the presence of the test compound is measured by comparing the incorporation of label in reaction product to a parallel reaction lacking the agent.

As those in the art will appreciate, other telomerase assays known in the art may also be adapted for use in conjunction with the practice of this invention. Moreover, the telomerase assays preferred for use in accordance with this invention are those which are scalable and thereby useful in high throughput screening (*e.g.*, to run in parallel from more than about 10 to about 10,000 or more separate reactions).

Screening for Telomerase Assembly and Activity Modulators

As described in detail in the Examples, *below*, the present invention is based on the unexpected discovery that the proteins p23 and hsp90 interact with TERT *in vitro* and *in vivo*, and that this association results in enhanced assembly of the TERT/TR complex resulting in active telomerase. One aspect of the present invention, based in part on this discovery, provides a method of screening for telomerase assembly inhibitors by incubating p23 (and/or other now known or later discovered telomerase chaparonins) and TERT with a test compound, comparing the association of p23 and TERT in the presence of the test compound, as compared to such association in the absence of the test compound, and identifying a compound that inhibits such association as a telomerase assembly inhibitor. In related embodiments, the invention provides methods of screening for telomerase assembly inhibitors by incubating hsp90 and TERT with test compound, comparing the association of hsp90 and TERT in the presence of the test compound with association in the absence of the test compound, and identifying a compound that inhibits the association of hsp90 and TERT as a telomerase assembly inhibitor.

Methods for measuring the association (*i.e.*, covalent or non-covalent binding or other intermolecular interaction) between two proteins are well known. The association may be measured *in vitro* (*e.g.*, using purified proteins in the presence or absence of the test compound), in cells (*e.g.*, incubated in the presence or absence of the

test compound), or in cell lysates (where the incubation with the test compound is carried out either in intact cells before lysis, or in the cell lysates themselves).

Any suitable method may be used in the present invention to measure the association between TERT telomerase chaparonins such as p23 or p90, including by gel filtration, gel-shift assays (also called electrophoretic/mobility shift assays), and matrix binding assays. Matrix binding assays include several variations to a basic technique in which one protein (*e.g.*, TERT) of a protein pair (*e.g.*, TERT and p23, or TERT and p90) is bound (*e.g.*, by UV cross-linking or by binding to an immobilized immunoglobulin) to a matrix (*e.g.*, a nylon, nitrocellulose, or plastic matrix) and the second protein, which is detectably labeled or otherwise detectable, is added under conditions (*e.g.*, pH, ionic strength, *etc.*) that permit the two proteins to associate. Residual unbound protein is removed by washing the membrane, and the amount of bound second protein is determined, *e.g.*, by quantitation of the detectable signal. By assaying the binding in the presence and absence of the test compound, it is possible to identify compounds that interfere with or inhibit the binding of the two proteins. One useful assay is described in the Examples, *below*.

In one embodiment of the invention, immunological assays, such as a "two antibody sandwich assay" are used. Such assays are well known in the art and are described, for example, in Harlow and Lane, ANTIBODIES: A LABORATORY MANUAL, COLD SPRING HARBOR LABORATORY, New York (1988), Chapter 14 (e.g., p. 583); and Ausubel et al., 1997, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing and Wiley-Interscience, New York. Briefly, in one method, an immobilized first antibody specific for one protein (e.g., TERT) in a heteromeric complex (e.g., p23/hsp90/TERT) is used to capture the first protein, a second protein (e.g., p23) is allowed to associate with the bound first protein in the presence or absence of a test compound, and the presence of the second protein is detected using an antibody specific for the second protein. As noted *supra*, numerous variations of these assays (e.g., differences in the order of addition components, the use of a non-immobilized first antibody that is immobilized after complex formation, etc.) as well as alternative assays and assay formats, are well known in the art. One of ordinary skill, once informed by the present disclosure of the association of p23 and hsp90 with the TERT protein, will be able without difficulty to design assays to detect agents that affect this association.

A Native Telomerase Complex in Cells Contains Both p23 and hsp90

As described in detail in the Examples, *below*, the present invention is based on the discovery that, unexpectedly, a native telomerase RNP complex in cells contains both p23 and hsp90. Thus, in one aspect, the invention provides methods of detecting an active telomerase complex by detecting TERT associated with p23 or hsp90, or both. Assays such as those described herein can be used to detect such complexes in lysates of telomerase positive cells. Quantitation of the TERT/hsp90/p23 complexes can be used for diagnosis and prognosis of telomerase-associated diseases.

Methods of Modulating Telomerase Activity

As demonstrated in the Examples, *below*, the present invention is based in part on the discovery that depletion of p23 from a reticulocyte lysate-based *in vitro* telomerase assembly reaction results in reduced telomerase activity in the reaction. Similarly, the inhibition of hsp90 function results in reduced telomerase activity. Thus, in certain embodiments of this aspect, the present invention provides methods and compositions for decreasing telomerase activity in a cell by decreasing the amount of one or more telomerase chaparonins, *e.g.*, hsp90 or p23, in the cell and/or inhibiting the association between such proteins and TERT. As those in the art will appreciate, inhibition of telomerase activity *in vitro* or *in vivo* may be used to treat diseases and disorders associated with cell immortality, such as neoplasia and pathogenic eukaryotic organisms.

Other aspects and embodiments concern stimulation of telomerase activity by enhancing such association to increase telomerase activity and extend telomere length, and will thereby be useful in the treatment of conditions associated with cellular senescence (*e.g.*, aging of tissues, organs, and organisms) or an increased rate of cell proliferation, such as is observed in cases of premature aging. For example, such stimulation would be useful to forestall and reverse cellular senescence, including but not limited to conditions associated with cellular senescence, *e.g.*, (a) cells with replicative capacity in the central nervous system, including astrocytes, endothelial cells, and fibroblasts which play a role in such age-related diseases as Alzheimer's disease, Parkinson's disease, Huntington's disease, and stroke, (b) cells with finite replicative capacity in the integument, including fibroblasts, sebaceous gland cells, melanocytes, keratinocytes, Langerhan's cells, and hair follicle cells which may play a role in age-

related diseases of the integument such as dermal atrophy, elastolysis and skin wrinkling, sebaceous gland hyperplasia, senile lentigo, graying of hair and hair loss, chronic skin ulcers, and age-related impairment of wound healing, (c) cells with finite replicative capacity in the articular cartilage, such as chondrocytes and lacunal and synovial fibroblasts which play a role in degenerative joint disease, (d) cells with finite replicative capacity in the bone, such as osteoblasts and osteoprogenitor cells which play a role in osteoporosis, (e) cells with finite replicative capacity in the immune system such as B and T lymphocytes, monocytes, neutrophils, eosinophils, basophils, NK cells and their respective progenitors, which may play a role in age-related immune system impairment, (f) cells with a finite replicative capacity in the vascular system including endothelial cells, smooth muscle cells, and adventitial fibroblasts which may play a role in age-related diseases of the vascular system including atherosclerosis, calcification, thrombosis, and aneurysms, and (g) cells with a finite replicative capacity in the eye such as pigmented epithelium and vascular endothelial cells which may play an important role in age-related macular degeneration. *See* WO 93/23572 for a more complete discussion of such diseases and conditions.

Reducing telomerase assembly/activity in a cell is useful, *inter alia*, to change the proliferative capacity of the cell. For example, reduction of telomerase activity in an immortal cell, such as a malignant tumor cell, will reduce the ability of the cell to proliferate. Decreasing the proliferative capacity of tumor cells will alleviate the disease or reduce the aggressive nature of a cancer to a more manageable disease state (increasing the efficacy of traditional interventions). *See, e.g.*, WO 98/14593. Some of the diseases which can be treated in accordance with these methods include cancers, of any type which have an elevated level of telomerase activity (*i.e.*, the absolute level of telomerase activity in the particular cell is elevated compared to normal cells in that individual, or compared to normal cells in other individuals not suffering from the condition), including solid tumors and leukemias (including those in which cells are immortalized, including: apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (*e.g.*, Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, *in situ*, Krebs 2, merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukemia (*e.g.*, b-cell, mixed-cell, null-cell, T-cell, T-cell chronic, HTLV-II-associated, lyphocytic acute, lymphocytic chronic, mast-cell, and myeloid), histiocytosis malignant,

Hodgkin's disease, immunoproliferative small, non-Hodgkin's lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocytoma, lipoma, liposarcoma, mesothelioma, myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing's sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, craniopharyngioma, dysgerminoma, hamartoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma, trophoblastic tumor, adenocarcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, leydig cell tumor, papilloma, sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, angiokeratoma, angiolymphoid hyperplasia with eosinophilia, angioma sclerosing, angiomatosis, glomangioma, hemangioendothelioma, hemangioma, hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangiomyoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes, fibrosarcoma, hemangiosarcoma, leiomyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma, myxosarcoma, ovarian carcinoma, rhabdomyosarcoma, sarcoma (*e.g.*, Ewing's, experimental, Kaposi's, and mast-cell), neoplasms (*e.g.*, bone, breast, digestive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous system, acoustic, pelvic, respiratory tract, and urogenital), neurofibromatosis, and cervical dysplasia), and for treatment of other conditions in which cells have become immortalized.

Other diseases, in addition to neoplasias wherein the tumor cells have acquired an immortal phenotype through the inappropriate activation of telomerase, including various human and veterinary parasitic diseases, can also be treated in accordance with the instant methods, particularly where the parasites or pathogens which cause the disease themselves express telomerase, as is the case for many pathogens that are eukaryotes. A representative sampling of some of these diseases include human protozoal pathogens such as; amebiasis from *Entamoeba histolytica,* amebic meningoencephalitis from the genus *Naegleria* or *Acanthamoeba,* malaria from *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae,* and *Plasmodium*

falciparum, Leishmaniasis from such protozoa as *Leishmania donovani*, *Leishmania infantum*, *Leishmania chagasi*, *Leishmania tropica*, *Leishmania major*, *Leishmania aethiopica*, *Leishmania mexicana*, and *Leishmania braziliensis*, Chagas' disease from the protozoan *Trypanosoma cruzi*, sleeping sickness from *Trypanosoma brucei*, *Trypanosoma gambiense*, and *Trypanosoma rhodesiense*, Toxoplasmosis from *Toxoplasma gondii*, giardiasis from *Giardia lamblia*, cryptosporidiosis from *Cryptosporidium parvum*, trichomoniasis from *Trichomonas vaginalis*, *Trichomonas tenax*, *Trichomonas hominis*, pneumocystis pneumonia from *Pneumocystis carinii*, bambesosis from *Bambesia microti*, *Bambesia divergens*, and *Bambesia boris*, and other protozoans causing intestinal disorders such as *Balantidium coli* and *Isospora belli*. Telomerase inhibitors would also be useful in treating certain helminthic infections including the species: *Taenia solium*, *Taenia saginata*, *Diphyllobothrium lata*, *Echinococcus granulosus*, *Echinococcus multilocularis*, *Hymenolepis nana*, *Schistosoma mansomi*, *Schistosoma japonicum*, *Schistosoma hematobium*, *Clonorchis sinensis*, *Paragonimus westermani*, *Fasciola hepatica*, *Fasciolopsis buski*, *Heterophyes heterophyes*, *Enterobius vermicularis*, *Trichuris trichiura*, *Ascaris lumbricoides*, *Ancylostoma duodenale*, *Necator americanus*, *Strongyloides stercoralis*, *Trichinella spiralis*, *Wuchereria bancrofti*, *Onchocerca volvulus*, *Loa loa*, *Dracunculus medinensis*, and fungal pathogens such as: *Sporothrix schenckii*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Aspergillus flavus*, *fungi of the genera Mucor and Rhizopus*, and species causing chromomycosis such as those of the genera *Phialophora and Cladosporium.* and important veterinary protozoal pathogens such as: *Babesia caballi*, *Babesia canis*, *Babesia equi*, *Babesia felis*, *Balantidium coli*, *Besnoitia darlingi*, *Eimeria acervulina*, *Eimeria adenoeides*, *Eimeria ahsata*, *Eimeria alabamensis*, *Eimeria auburnensis*, *Eimeria bovis*, *Eimeria brasiliensis*, *Eimeria brunetti*, *Eimeria canadensis*, *Eimeria cerdonis*, *Eimeria crandallis*, *Eimeria cylindrica*, *Eimeria debliecki*, *Eimeria despersa*, *Eimeria ellipsoidalis*, *Eimeria fauvei*, *Eimeria gallopavonis*, *Eimeria gilruthi*, *Eimeria granulosa*, *Eimeria hagani*, *Eimeria illinoisensis*, *Eimeria innocua*, *Eimeria intricata*, *Eimeria leuskarti*, *Eimeria maxima*, *Eimeria meleagridis*, *Eimeria meleagrimitis*, *Eimeria mitis*, *Eimeria mivati*, *Eimeria necatrix*, *Eimeria neodebliecki*, *Eimeria ninakohlyakimorae*, *Eimeria ovina*, *Eimeria pallida*, *Eimeria parva*, *Eimeria perminuta*, *Eimeria porci*, *Eimeria praecox*, *Eimeria punctata*, *Eimeria scabra*, *Eimeria spinoza*, *Eimeria subrotunda*,

Eimeria subsherica, Eimeria suis, Eimeria tenella, Eimeria wyomingensis, Eimeria zuernii, Endolimax gregariniformis, Endolimax nana, Entamoeba bovis, Entamoeba gallinarum, Entamoeba histolytica, Entamoeba suis, Giardia bovis, Giardia canis, Giardia cati, Giardia lamblia, Haemoproteus meleagridis, Hexamita meleagridis, Histomonas meleagridis, Iodamoeba buetschili, Isospora bahiensis, Isospora burrowsi, Isospora canis, Isospora felis, Isospora ohioensis, Isospora rivolta, Isospora suis, Klossiella equi, Leucocytozoon caallergi, Leucocytozoon smithi, Parahistomonas wenrichi, Pentatrichomonas hominis, Sarcocystis betrami, Sarcocystis bigemina, Sarcocystis cruzi, Sarcocystis fayevi, hemionilatrantis, Sarcocystis hirsuta, Sarcocystis miescheviana, Sarcocystis muris, Sarcocystis ovicanis, Sarcocystis tenella, Tetratrichomonas buttreyi, Tetratrichomonas gallinarum, Theileria mutans, Toxoplasma gondii, Toxoplasma hammondi, Trichomonas canistomae, Trichomonas gallinae, Trichomonas felistomae, Trichomonas eberthi, Trichomonas equi, Trichomonas foetus, Trichomonas ovis, Trichomonas rotunda, Trichomonas suis, and *Trypanosoma melophagium.*

Reducing Telomerase Chaparonin Levels in a Cell

The quantity of hsp90 and/or p23 protein (or one or more other telomerase chaparonins) in a cell can be reduced by a variety of methods known in the art to result in a decrease in telomerase activity in a cell (and an associated decrease in telomere length and cell proliferative capacity). Typically, the level of a protein in a cell is reduced by interfering with the expression of the gene encoding the protein (*e.g.*, by reducing transcription, RNA processing or stability, or translation). Exemplary methods include, but are not limited to, the use of antisense, triplex, or ribozyme polynucleotides (together referred to herein as "inhibitory nucleic acids") to reduce expression of one or telomerase chaparonins, alone or in conjunction with reducing expression of HERT and/or TR. For example, antisense oligonucleotides or polynucleotides complementary to the nucleotide sequence of the target (*e.g.*, human) p23 or hsp90 gene sequence (described elsewhere herein) can be expressed in, or administered to, cells to decrease hsp90/p23 levels (*see, e.g.*, Milner, *et al.*, 1997, *Nature Biotechnology* 15:537; Uhlmann et al., 1990, *Chem. Reviews*, 90:543-584). Without intending to be limited to any particular mechanism, it is believed that antisense oligonucleotides bind to, and interfere with the translation or stability of the target mRNA. Alternatively, for example, telomerase chaparonin-specific

ribozymes, for example, targeted to hsp90 and/or p23, comprising 5'- and 3'-terminal sequences complementary to target mRNA sequences can be engineered (*see* PCT publications WO 93/23572, WO 94/02595, and WO 93/23569) and administered to a cell. Methods useful for delivery of nucleic acids, including oligonucleotides, ribozymes, and expression vectors encoding one or more of such molecules (alone or in combination) to cells are known in the art (*e.g.*, including introduction into the cell using liposomes, immunoliposomes, ballistics, direct uptake into cells, viral vectors, any of the foregoing of which may further comprise cell- or tissue-specific targeting molecules, and the like; *see*, *e.g.*, U.S. Patent 5,272,065).

Inhibiting the Association Between TERT and Telomerase Chaparonins

In a related aspect, the present invention provides methods and compositions for decreasing telomerase activity in a cell by inhibiting the association between a telomerase chaparonin, *e.g.*, p23 or hsp90, and a component of the telomerase holoenzyme (which comprises the telomerase catalytic protein component in association with the telomerase RNA component). In some embodiments the inhibitor is a molecule identified in a modulator screen as described herein.

In another embodiment, the telomerase component/telomerase chaparonin interaction is inhibited by a molecule (*e.g.*, a small, synthetic organic molecule or peptide) that interacts with the site in the amino terminal domain of TERT that binds p23 (*e.g.*, a site within the amino-terminal 195 residues of hTERT). As those in the art will appreciate, the HERT and/or telomerase chaparonin components useful in the practice of this invention include functionally active proteins and variants (*e.g.*, those having one or more amino acid changes as compared to the native amino acid sequence(s) then known for the corresponding polypeptide), inactive variants, and nucleic acids encoding the same. In addition, due to the degenerate nature of the genetic code, the nucleic acids used in practicing various aspects this invention need not be those of the corresponding naturally occurring genes, but instead can be any nucleic acid coding for an RNA or amino acid sequence, as the case may be, of the desired expression product.

In still another embodiment, a benzoquinone ansamysin, such as geldanamycin, is used to inhibit telomerase assembly and activity.

Compositions

Certain aspects of this invention concern pharmaceutical or veterinary compositions comprising one or more telomerase modulators according to the invention, alone or in combination with at least one other agent, such as a stabilizer, diluent, carrier, excipient, adjuvant or other ingredient not capable of eliciting the desired therapeutic effect. The compounds of the invention are preferably delivered in any sterile, biopharmaceutical carrier, including, but not limited to saline, buffered saline, dextrose, and water, and can be delivered alone or in combination with other therapeutic agents, drugs, or hormones.

For any compound identified and used in the practice of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. Such information can be used to more accurately determine useful doses in organisms such as plants and animals, preferably mammals, and most preferably humans. Levels in plasma may be measured, for example, by HPLC or other means appropriate for detection of the particular compound.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition (see *e.g.* Fingl *et. al.*, in The Pharmacological Basis of Therapeutics, 1975, Ch. 1 p.1).

It should be noted that the attending physician would know how and when to terminate, interrupt, or adjust administration due to toxicity, organ dysfunction, or other systemic malady. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated and the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above also may be used in veterinary applications of the invention.

Depending on the specific diseases state being treated and the method selected, such agents may be formulated and administered systemically or locally, *i.e.*, topically. Techniques for formulation and administration may be found in Alfonso and Gennaro (1995). Suitable routes may include, for example, oral, rectal, transdermal,

vaginal, transmucosal, intestinal, parenteral, intramuscular, subcutaneous, or intramedullary injections, as well as intrathecal, intravenous, or intraperitoneal injections.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable carriers to formulate identified anti-microbials of the present invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular those formulated as solutions, may be administered parenterally, such as by intravenous injection. Appropriate compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Further details on formulation, compounding, and administration techniques can be found in the latest edition of "Remington's Pharmaceutical Sciences," Maack Publishing Co., Easton PA. Compositions according to the invention include those in solution or dry formulations.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions, including those formulated for delayed release or only to be released when the pharmaceutical reaches the small or large intestine.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Alternatively, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to

the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

Miscellaneous Methods

I. Molecular Biological Techniques

Methods useful for cloning, expression and manipulation of nucleic acids encoding telomerase assembly associated proteins are well known in the art and are described, for example, in Sambrook et al., 1989, MOLECULAR CLONING: A LABORATORY MANUAL, 2ND ED., VOLS. 1-3, Cold Spring Harbor Laboratory, and Ausubel et al., 1997, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing and Wiley-Interscience, New York.

II. Determination of Substantial Identity Between Sequences

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

The phrase "substantially identical," in the context of two nucleic acids or polypeptides, refers to two or more sequences or subsequences that have at least 60%, preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues. In a most

preferred embodiment, the sequences are substantially identical over the entire length of the coding regions.

EXAMPLES

The following examples are offered for purposes of illustration of various aspects of the invention, and are limiting in any way.

Example 1: The Phosphoprotein p23 Interaction with hTERT

A yeast two-hybrid system (Fields, *et al.*, 1989, *Nature* 340:245-246) was used to screen for molecules that interact with the human telomerase reverse transcriptase (hTERT) protein using the LexA-dependent reporter strain L40 and a mouse embryo library (Vojtek et al., 1993, *Cell* 74:205-214).

The "bait" used in the screen was a gene encoding the N-terminal 195 residues of hTERT with codon usage optimized for expression in *S. cerevisiae.* The gene was assembled from six oligonucleotide pairs (HHMI/Keck Oligonucleotide Facility, Yale Univ.), the ends of which were staggered by ten bases to create complementary sequences for ligation. The oligonucleotides were gel purified. Each of the pairs was generated by annealing of gel purified oligonucleotides by heating to 100°C for 10', then at 90°C for 10', followed by rapid chilling. The full-length construct (pJBT1) was built by sequential ligation of adjoining pairs with gel purification of ligated fragments prior to each subsequent ligation. The final product was subcloned into pJBT0, a modified form of pBluescript II-SK+ (Stratagene). The BamHI-SalI fragment of pJBT1 was inserted into pBTM116 (Vojtek et al., *supra*) to create the bait encoding the first 195 amino acids of hTERT.

Transformants, from the yeast reporter strain L40, selected to express LexA-hTERT (1-195) and VP16-p23 alone or together were tested for the ability to grow on media lacking histidine. Growth on the selective plate indicated a positive two-hybrid interaction (Johnson et al., 1994, *Mol. Cell. Bio.* 14:1956-1963). The screens of the mouse embryo library of cDNAs expressed as fusions to the VP16 activation domain against a bait expressing the amino terminus of hTERT (amino acids 1-195) fused to the LexA DNA-binding domain resulted in the isolation of a clone encoding a 23 KDa phosphoprotein, p23.

Example 2: hTERT Associates with p23 and hsp90 *In Vitro*

Together with the heat shock protein hsp90, p23 has been implicated in mediating formation of receptor/ligand complexes (Pratt et al., 1997, *Endocrine Reviews* 18:306-360) as well as a protein/RNA complex (Hu et al., 1997, *EMBO* 16:59-68). To determine whether the hTERT/p23 interaction identified by yeast two-hybrid analysis played a role in the assembly of *in vitro* translated hTERT and the template RNA (hTR) into active complexes, the association of *in vitro* transcribed and translated hTERT with p23 and hsp90 was assayed.

hTERT was synthesized in the rabbit reticulocyte system (Promega) as previously described (Weinrich *et al.*, 1997, *Nature Gen.* 17: 498-502) in the presence of ^{35}S-methionine. hTERT cDNA was cloned into pcDNA3.1/HisC (Invitrogen) with the addition of a sequence encoding three copies of a carboxy-terminal hemagluttinen epitope (HA). For immunoprecipitation, ^{35}S-labeled hTERT was incubated with normal mouse IgG, the monoclonal anti-p23 antibody JJ3 (Johnson et al., 1994, *Mol. Cell. Bio.* 14:1956-63), the monoclonal anti-hsp90 antibody H9010, or monoclonal anti-HA (12CA5, Boehringer Mannheim), at a final antibody concentration of 0.5 μg/ml for one hour on ice. Protein G agarose (Boehringer Mannheim) was added and the mix was incubated at 4°C with constant rotation. Agarose pellets were subsequently washed three times with 20mM HEPES (pH 7.6), 20% glycerol, 100mM NaCl, 0.2mM EGTA, 1mM $MgCl_2$, 0.1% NP-40, and 0.1% BSA. Washed pellets were heated to 80°C for 10 minutes and electrophoresed on SDS-PAGE (7.5%). 10% of the total translation reaction was loaded to reveal the amount of labeled hTERT present in the reticulocyte lysates. The presence of co-precipitated hTERT was determined by phosphorimager analysis by exposing dried gels to a phosphorimage screen (Molecular Dynamics) for 24-48 hours. The amount of hTERT precipitated with anti-p23 and anti-hsp90 antibodies was comparable to that directly precipitated with the 12CA5 anti-HA antibody.

The ^{35}S-methionine labeled hTERT synthesized in rabbit reticulocyte lysate was immunoprecipitated with either anti-p23 or anti-hsp90 antibodies, demonstrating that full-length hTERT can associate with these molecules *in vitro*. This association did not require the presence of the telomerase template RNA.

Example 3: A Native Telomerase Complex in Cells Contains Both p23 and hsp90

The interaction observed in reticulocyte lysates was also observed in human tumor cells. For example, telomerase activity was present in anti-p23 but not normal mouse IgG immunoprecipitates from HT1080 fibrosarcoma cells. Hsp90 antibodies also coimmunopreciptated telomerase activity, demonstrating that a native telomerase complex in cells contains both p23 and hsp90. To carry out these experiments, HT1080 cells were cultured in 10% serum on 15 cm plates. After reaching confluence, one plate of cells per immunoprecipitation was lysed by douncing in ice-cold PBS with protease inhibitors. Ten µl of antibody together with 30 µl protein A/G agarose were added to the lysates (1ml) and incubated at 4°C for 1 hour. Pellets were washed 4 times, 10 minutes each time, in lyses buffer. Aliquots were then removed and assayed directly in TRAP as previously described (Holt et al., 1997, *Proc. Natl. Acad. Sci.* 94:10687-692). Normal mouse IgG was used as a control for specificity.

The isolation of p23 and Hsp90 telomerase complexes followed by telomerase activity assays as just described can be used for various purposes, including for use in diagnosis and prognosis of telomerase-related diseases.

Example 4: p23 Promotes Assembly of Telomerase Activity *In Vitro*

The functional importance of the p23 and hsp90 association with hTERT was shown by immunodepletion studies. The experiments were carried out as follows: *in vitro* transcribed and translated hTERT was diluted 1/20 into (1) fresh rabbit reticulocyte lysate ("+RRL"), (2) p23 depleted RRL (immunodepletion of RRL with anti-p23 was performed as previously described in Johnson et al., 1994, *J. Biol. Chem.* 269:24989-993), (3) p23 depleted RRL (p23 dep RRL) supplemented with 0 ng, 50 ng or 100 ng recombinant p23, or (4) a buffer comprising 10 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, pH 7.5, ("-RRL"). The hTERT/RRL mixture was mixed with 0.5 µg of *in vitro* transcribed hTR. The mixture was incubated at 30°C for 90 minutes to allow assembly of hTERT and its hTR template. An aliquot was then removed and assayed for activity by TRAP. Depletion of p23 from RRL was verified by Western analysis using the anti-p23 antibody. Quantitation was performed by densitometry analysis of TRAP assay versus the internal control as described (Holt et al., 1997, *Proc. Natl. Acad. Sci. USA* 94:10687-10692). The results of the experiment are summarized in Table 1.

TABLE 1

Reaction	Telomerase activity (arbitrary units)
-RRL	0.1
+RRL	10
p23dep RRL/+ 0 ng p23 (no addition)	0.6
p23dep RRL/ +50 ng p23	2.3
p23dep RRL/+ 100 ng p23	3

Addition of reticulocyte lysate to the hTERT/hTR reconstitution step ("+RRL") resulted in a 50 to 100-fold increase in telomerase activity consistent with the observation that factors in rabbit reticulocyte lysate promote the efficient assembly of in vitro translated hTERT and the template RNA (hTR) into active complexes. This increased activity is independent of additional protein synthesis, as it was insensitive to addition of cyclohexamide (Weinrich et al., 1997, *Nature Gen.* 17: 498-502). However, immunodepletion of p23 from reticulocyte lysate significantly decreases its ability to stimulate generation of telomerase activity. Addition of purified recombinant p23 to the immunodepleted reticulocyte lysate restores activity, demonstrating that p23 promotes assembly of telomerase activity *in vitro*. Failure to fully generate activity with purified p23 may be due to co-depletion of p23-associated factors or incompletely active purified protein.

Example 5: The hsp90 Inhibitor Geldanamycin Blocks the Enhancement of Telomerase Reconstitution *In Vitro* by Reticulocyte Lysate

The benzoquinone ansamysin, geldanamycin, inhibits some functions of hsp90 by binding to its ATP-binding site, and it also blocks ATP-dependent binding of p23 to hsp90 (Grenert et al., 1997, *J. Biol. Chem.* 272:23843-850; Prodromou et al., 1997,

Cell 90:65-75). Incubation of reticulocyte lysates with geldanamycin (Calbiochem) completely blocked the ability of reticulocyte lysates to enhance telomerase assembly (as assessed by the TRAP assay). In this experiment, telomerase was assembled as described above, except that translated hTERT was diluted in RRL that had been incubated in the presence of geldanamycin (100ug/ml in a final DMSO concentration of 10%) or DMSO only (10%) carrier for 30 minutes. Addition of geldanamycin (100 μg/ml) after the assembly step, but prior to the telomerase activity assay, had no effect on activity. Thus, thus geldanamycin did not inhibit association of hTERT/hTR with the substrate primer or inhibit enzymatic activity *in vitro*.

Example 6: Geldanamycin Does Not Block Binding of hTR to hTERT

Geldanamycin does not exert its effects by blocking hTR binding by hTERT This was demonstrated by experiments in which ^{35}S-labeled HA-tagged hTERT synthesized in the presence of geldanamycin or DMSO carrier was assayed for association with immobilized hTR versus control human U2 snRNA (U2).

Briefly, full-length hTR or human U2 snRNA were transcribed from pTRC3 and pGEM-U2 respectively using T7 RNA polymerase (Weinrich et al., 1997, *Nature Gen.* 17:498-502). The resulting transcripts contain a 90 base linker sequence corresponding to the pGEM-5Zf(+) polylinker. This linker hybridizes with a complementary biotinylated oligonucleotide, allowing for retrieval of ribonucleoprotein complexes with streptavidin magnetic beads. ^{35}S-labeled hTERT was translated in RRL in the presence of 50 μg/ml geldanamycin or 5% DMSO (carrier). 2.5 μg of hTR or U2 RNA, bound to 100 pmols biotinylated oligonucleotide, was incubated with ^{35}S-labeled-hTERT and *in vitro* synthesized ^{35}S-labeled luciferase (used as an internal control for specificity) for one hour at 30°C. Proteins bound to beads were analyzed by 7.5% SDS-PAGE and autoradiography. Northern analysis was used to confirm that linker-hTR and linker-U2 were captured in all reactions. An aliquot (10% of input) of each translation reaction was analyzed for comparison of amounts hTERT and luciferase present. The addition of geldanamycin had no effect on the association of hTERT and hTR.

These results, taken with those in the sections *supra*, are consistent with hsp90/p23 activity not being required for hTERT to bind hTR, but acting to facilitate formation of a functional hTERT/hTR complex.

Example 7: Geldanamycin Inhibits hsp90/p23 Activity in Intact Cells

The functional importance of p23 and hsp90 in hTERT complexes in intact cells was established using geldanamycin to inhibit hsp90/p23 activity. Telomerase activity in human fibrosarcoma cells (HT1080) is downregulated when the cells become quiescent and is re-induced upon growth stimulation (Holt et al., 1997, *Proc. Natl. Acad. Sci. USA* 94:10687-692). Serum-starved, quiescent HT1080 cells with low levels of telomerase activity were first treated with a range of concentrations of geldanmycin or the carrier DMSO, followed by addition of serum. HT1080 cells were cultured as described previously (Holt et al., 1997, *Proc. Natl. Acad. Sci. USA* 94:10687-692). Cells were incubated in the absence of serum for 14 days. Serum was then added back to the cultures with one of the following additions: DMSO, geldanamycin, FK506 (gift from Fujisawa USA, Inc.), or Cyclosporin A (Sigma), for 24 hours. 24 hours after exposure to serum, cells were lysed and assayed for telomerase activity by TRAP. Cells treated with carrier alone expressed high levels of telomerase activity 24 hours post serum stimulation. In contrast, cells treated with geldanmycin at concentrations of 100 ng/ml or greater failed to express active telomerase in response to serum. Thus, exposure of cells to an inhibitor of hsp90 blocks induction of telomerase activity.

Geldanamycin blocked induction of telomerase activity at concentrations that did not affect S-phase entry as determined by ^{3}H-thymidine incorporation assays (Holt et al., 1996, *Mol. Cell. Biol.* 16:2932-39. Short-term viability was also not affected. Effects on plating efficiency were assayed by cell counts before and after stimulation followed by replating. All samples had similar plating efficiencies of 70-80% except for treatment with 40 μg/ml of CsA which reduced plating efficiencies to 60%.

p23 and hsp90 have been reported to exist in complexes containing one or more immunophilins including FK506-binding protein and cyclosporin A (Pratt et al., 1997, *Endocrine Reviews* 18:306-360; Freeman et al., 1996, *Science* 274:1718-1720). Incubation of HT1080 cells with FK506 (5 μM or 50 μM) or cyclosporin (10 μg/ml or 40 μg/ml), at concentrations that blocked induction of DNA synthesis or were moderately toxic over the 24-hour incubation, did not affect induction of telomerase activity. These results are consistent with a requirement for p23 and hsp90 but not immunophilin function for induction of active telomerase *in vivo*.

Example 8: Enhancement of Telomerase Activity by Addition of Heat Shock Proteins 40, 70 and 90; HOP, and p23.

Substitution of purified Hsp90 and p23 for RRL in an *in vitro* telomerase assembly reaction did not enhance the production of active telomerase. However, when Hsp70, HOP, and Ydj-1 (yeast homolog of Hsp40) were added to the assembly reaction with Hsp90 and p23, telomerase activity was comparable to assembly reactions containing RRLs. Further, supplementing the rabbit reticulocyte lysate with all five purified recombinant telomerase assembly associated proteins (hsp 40, hsp70, hsp90, HOP, and p23) at the appropriate ratios, as described below, results in a 5-10 fold increase in the enhancement of telomerase reconstitution over reticulocyte lysate alone.

In vitro telomerase assembly assays were performed using the following protocols:

To assemble active telomerase, 0.2 µl of *in vitro*-transcribed and -translated hTERT and 0.5 µg of hTR were mixed together in a 4 µl assembly assay with or without additional fresh RRL. The reaction was mixed and incubated for 90 minutes at 30°C.

Protocol for Reconstitution of Telomerase from Purified Proteins

A 5 µL reconstitution is first prepared from the following components added in order:

PCR grade water	bring the total volume to 5 µL
10X buffer (see below)	0.5 µL
p23	125 ng
hsp90	750 ng
hsp70	500 ng
Hop (p60)	25 ng
YDJ (hsp40 homologue)	25 ng
ATP	5 mM final concentration
hTERT (from TNT)	0.2 µL (of the TNT reaction)
hTR (from T7 Megascript)	0.5 µL (1.0 µg/µL)

*Buffer for the reconstitution reaction is 10 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$ and 2 mM dithiothreitol, pH 7.5.

To test for telomerase activity, the total volume is then brought up to 100 μL with CHAPS Lysis Buffer and 1 μL of this diluted sample replaces the usual 2 μL sample in a regular TRAP assay.

Addition of any subset of these components does not result in an equivalent enhanced activity. These results indicate that hsp70, hsp40, and HOP activity are all required for association of hsp90 and p23 with telomerase.

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All publications and patent documents cited in this application are explicitly incorporated by reference in their entirety and for all purposes to the same extent as if each individual publication or patent document were so individually denoted. While the instant invention has been described above both in generic terms and in terms of preferred embodiments, variations will be apparent to those skilled in the art, and the invention should not be construed as limited to the specific embodiments described above, but instead is as set forth in the claims that follow.

WHAT IS CLAIMED IS:

1. Method of assembling an active telomerase *in vitro* comprising combining in a reaction mixture a telomerase catalytic protein component, a telomerase RNA component, and a telomerase chaparonin.

2. A method according to claim 1 wherein at least one of the telomerase catalytic protein component, the telomerase RNA component, and a telomerase chaparonin are transcribed and/or translated in the reaction mixture.

3. A method according to claim 1 wherein each of the telomerase catalytic protein component, the telomerase RNA component, and the telomerase chaparonin are added to the reaction mixture are produced by recombinant methods.

4. A method according to claim 1 wherein at least one of the telomerase catalytic protein component, the telomerase RNA component, and a telomerase chaparonin are added to the reaction mixture in substantially purified form.

5. A method according to claim 1 at least one of the telomerase catalytic protein component, the telomerase RNA component, and a telomerase chaparonin are derived from a mammalian source.

6. A method according to claim 1 wherein each of the telomerase catalytic protein component, the telomerase RNA component, and the telomerase chaparonin are derived from a human source.

7. A method according to claim 6 wherein each of the telomerase catalytic protein component, the telomerase RNA component, and the telomerase chaparonin comprise amino acid or nucleotide sequences substantially homologous to those found in nature.

8. A method according to claim 1 wherein the reaction mixture comprises more than one telomerase chaparonins.

9. A method according to claim 1 wherein the reaction mixture contains a telomerase chaparonin selected from the group consisting of p23, hsp40, hsp70, hsp90, and HOP proteins.

10. A method according to claim 9 wherein the telomerase chaparonin has been substantially purified prior to inclusion in the reaction mixture.

11. A method according to claim 9 wherein the telomerase chaparonin is a recombinant telomerase chaparonin.

12. Method of modulating assembly of active telomerase, the method comprising contacting a compound that modulates interaction of a telomerase chaparonin with a telomerase catalytic protein component or a telomerase RNA component, thereby modulating assembly of active telomerase.

13. A method according to claim 12 wherein the compound inhibits interaction of a telomerase chaparonin with the telomerase catalytic protein component or the telomerase RNA component.

14. A method according to claim 13 wherein the compound is selected from the group consisting of a small molecule, a nucleic acid, and a polypeptide.

15. A method according to claim 13 wherein the compound comprises a nucleic acid selected from the group consisting of an expression vector, an antisense nucleic acid, an oligonucleotide, and a ribozyme.

16. A method according to claim 12 wherein the compound stimulates interaction of a telomerase chaparonin with the telomerase catalytic protein component or the telomerase RNA component.

17. A method according to claim 16 wherein the compound is selected from the group consisting of a small molecule, a nucleic acid, and a polypeptide.

18. A method according to claim 12 wherein the contacting of the compound that modulates interaction of a telomerase chaparonin with the telomerase catalytic protein component or the telomerase RNA component occurs *in vitro*.

19. A method according to claim 12 wherein the contacting of the compound that modulates interaction of a telomerase chaparonin with the telomerase catalytic protein component or the telomerase RNA component occurs in a cell.

20. A method according to claim 19 wherein the cell is in culture.

21. A method according to claim 19 wherein the cell is *in vivo*.

22. Method of modulating telomerase activity, the method comprising contacting a compound that modulates interaction of a telomerase chaparonin with a telomerase catalytic protein component or a telomerase RNA component, thereby modulating telomerase activity.

23. A method according to claim 22 wherein the compound inhibits interaction of a telomerase chaparonin with the telomerase catalytic protein component or the telomerase RNA component.

24. A method according to claim 23 wherein the compound is selected from the group consisting of a small molecule, a nucleic acid, and a polypeptide.

25. A method according to claim 23 wherein the compound comprises a nucleic acid selected from the group consisting of an expression vector, an antisense nucleic acid, an oligonucleotide, and a ribozyme.

26. A method according to claim 22 wherein the compound stimulates interaction of a telomerase chaparonin with the telomerase catalytic protein component or the telomerase RNA component.

27. A method according to claim 26 wherein the compound is selected from the group consisting of a small molecule, a nucleic acid, and a polypeptide.

28. A method according to claim 22 wherein the contacting of the compound that modulates interaction of a telomerase chaparonin with the telomerase catalytic protein component or the telomerase RNA component occurs *in vitro*.

29. A method according to claim 22 wherein the contacting of the compound that modulates interaction of a telomerase chaparonin with the telomerase catalytic protein component or the telomerase RNA component occurs in a cell.

30. A method according to claim 29 wherein the cell is in culture.

31. A method according to claim 29 wherein the cell is *in vivo*.

32. Method of screening for a telomerase activity modulator, the method comprising:

a) combining in a reaction mixture a telomerase catalytic protein component, a telomerase RNA component, a telomerase chaparonin, and a test compound under conditions which, in the absence of the test compound, do not modulate telomerase activity;

b) determining whether the test compound modulates telomerase activity; and if so,

c) identifying the test compound as a telomerase activity modulator.

33. A method according to claim 32 used to identify an inhibitor of telomerase activity.

34. A method according to claim 32 used to identify a stimulator of telomerase activity.

35. Method of screening for a telomerase assembly inhibitor, the method comprising:

a) incubating a telomerase catalytic protein component and a telomerase chaparonin with a test compound;

b) comparing the association of the telomerase catalytic protein component and the telomerase chaparonin in the presence of the test compound with association of the telomerase catalytic protein component and the telomerase chaparonin in the absence of the test compound; and

c) identifying a test compound that decreases the association of the telomerase catalytic protein component and the telomerase chaparonin as a telomerase assembly inhibitor.

36. A method according to claim 35 wherein the telomerase chaparonin is selected from the group consisting of p23, hsp90, hsp40, hsp70, and HOP.

37. A method according to claim 35 wherein the telomerase catalyitc protein component and test compound are incubated with at least two telomerase chaparonins.

38. Method of modulating telomerase activity in a cell expressing a telomerase catalytic protein component and a telomerase RNA component, the method comprising a method selected from the group consisting of:

a) decreasing the amount of a telomerase chaparonin in the cell;

b) inhibiting association of a telomerase chaparonin with a telomerase catalytic protein component;

c) inhibiting association of a telomerase chaparonin with a telomerase RNA component;

d) stimulating association of a telomerase chaparonin with a telomerase catalytic protein component;

e) stimulating association of a telomerase chaparonin with a

telomerase RNA component; and

f) increasing the amount of a telomerase chaperonin in the cell,

in each instance through administration of an amount of a compound effective to modulate telomerase activity of the cell.

39. A method according to claim 38 wherein the cell is a vertebrate cell.

40. A method according to claim 38 wherein the cell is a animal cell selected from the group consisting of avian, bovine, canine, equine, feline, ovine, and porcine cells.

41. A method according to claim 38 wherein the cell is a human cell.

42. A method according to claim 38 wherein the cell is in culture.

43. A method according to claim 38 wherein the cell is *in vivo*.

44. A method according to claim 38 wherein the compound is selected from the group consisting of a small molecule, a nucleic acid, and a polypeptide.

45. A method according to claim 38 wherein the compound comprises a nucleic acid selected from the group consisting of an expression vector, an antisense nucleic acid, an oligonucleotide, and a ribozyme.

46. A pharmaceutical composition comprising a therapeutically effective amount of a compound which modulates telomerase activity through modulation of association between a telomerase chaperonin and a telomerase holoenzyme comprising a telomerase catalytic protein component and a telomerase RNA component.

47. A method according to claim 46 wherein the compound is selected from the group consisting of a small molecule, a nucleic acid, and a polypeptide.

48. A method according to claim 46 wherein the compound comprises a nucleic acid selected from the group consisting of an expression vector, an antisense nucleic acid, an oligonucleotide, and a ribozyme.

49. Method of modulating proliferation of cells comprising administering to cells which contain telomeres an amount of a compound which modulates telomerase activity through modulation of association between a telomerase chaparonin and a telomerase holoenzyme comprising a telomerase catalytic protein component and a telomerase RNA component.

50. A method according to claim 49 for treatment of a disease associated with an elevated level of telomerase activity, wherein the compound inhibits association between a telomerase chaparonin and a telomerase holoenzyme.

51. A method according to claim 50 wherein the disease is selected from the group consisting of cancer, leukemia, and infection with a eukaryotic pathogen.

52. A method according to claim 49 for increasing proliferative capacity of a cell, wherein the compound stimulates association between a telomerase chaparonin and a telomerase holoenzyme.

53. A method for stimulating telomerase activity in cells comprising administering to such cells an amount of a compound which increases telomerase activity by enhancing the association between a telomerase chaparonin and a telomerase holoenzyme comprising a telomerase catalytic protein component and a telomerase RNA component.

54. A method according to claim 53 for treatment of a condition associated with cellular senescence.

55. A method according to claim 54, wherein said condition is selected from the group consisting of Alzheimer's disease, Parkinson's disease,

Huntington's disease, hairloss, degenerative joint disease, macular degeneration and stroke.

56. A method according to claim 53 for treatment of conditions associated with cells with finite replicative capacities.

57. A method according to claim 56 wherein said cells are selected from the group consisting of hair follicle cells, chondrocytes, lacunal cells, synovial fibroblasts, osteoblasts, and osteoprogenitor cells.

58. A method according to claim 56 wherein said cells are selected from the group consisting of B lymphocytes, T lymphocytes, monocytes, neutrophils, eosinophils, basophils, NK cells and NK cell progenitors.

59. A method according to claim 56 wherein said cells are selected from the group consisting of pigmented epithelium and vascular endothelial cells.

60. An assay used for diagnosis and prognosis of telomerase-associated diseases comprising:

isolating TERT/hsp90/p23 complexes from cells;

quantitating telomerase activity of said complexes; and

determining if said activity is above or below normal.

61. A method according to claim 23 wherein the compound is Geldanamycin.

62. A method according to claim 30 wherein said cells are HT1080.

INTERNATIONAL SEARCH REPORT

Inter, ›nal Application No
PCT/US 99/17724

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/12 C12Q1/48 G01N33/573 G01N33/50 A61K38/45

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WEINRICH ET AL.: "RECONSTITUTION OF HUMAN TELOMERASE WITH THE TEMPLATE RNA COMPONENT HTR AND THE CATALYTIC PROTEIN SUBUNIT HTRT" NATURE GENETICS, vol. 17, December 1997 (1997-12), pages 498-502, XP002107652 ISSN: 1061-4036 cited in the application the whole document ---	1-7
A	WO 98 01542 A (UNIV CALIFORNIA) 15 January 1998 (1998-01-15) cited in the application page 2, line 18 - line 31; claim 12 --- -/--	1-7,12, 22,32, 35,46, 53,60

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 November 1999	08/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Ceder, O

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter. ...al Application No
PCT/US 99/17724

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NAKAMURA ET AL.: "TELOMERASE CATALYTIC SUBUNIT HOMOLOGS FROM FISSION YEAST AND HUMAN" SCIENCE, vol. 277, 15 August 1997 (1997-08-15), pages 955-959, XP002056803 ISSN: 0036-8075 cited in the application page 958, right-hand column	32,35, 49-52
A	MEYERSON M ET AL: "HEST2, THE PUTATIVE HUMAN TELOMERASE CATALYTIC SUBUNIT GENE, IS UP-REGULATED IN TUMOR CELLS AND DURING IMMORTALIZATION" CELL,US,CELL PRESS, CAMBRIDGE, NA, vol. 90, no. 4, 22 August 1997 (1997-08-22), pages 785-795, XP002056804 ISSN: 0092-8674 cited in the application abstract page 786 page 790, right-hand column	49-52
P,X	HOLT ET AL.: "Functional requirements of p23 and Hsp90 in telomerase complexes" GENE AND DEVELOPMENT, vol. 13, no. 7, 1 April 1999 (1999-04-01), pages 817-826, XP002122595 the whole document	1-62

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/17724

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 12-17, 19, 21-27, 29, 31, 38-41, 43-45 and 49-59 (as far as they concern in vivo methods) are directed to methods of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

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Continuation of Box I.2

Present claims 12-31 and 38-59 relate to a compound defined by reference to a desirable characteristic or property, namely modulation (inhibition or stimulation) of telomerase assembly or activity. The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compounds by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds indicated on page 23 lines 18-31 and in claim 61, i.e. compounds binding to the amino terminal domain of TERT that binds p23 and benzoquinone anamysin componds, e.g. geldanamycin.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 99/17724

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9801542 A	15-01-1998	US 5770422 A	23-06-1998
		AU 3728197 A	02-02-1998
		US 5917025 A	29-06-1999